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Hypoxia alters contractile protein homeostasis in L6 myotubes

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ABSTRACT

Since hypoxia might contribute to the development of muscle atrophy, we wished to provide direct evidence linking hypoxia to muscle atrophy. By evaluating protein degradation and synthesis in hypoxic myotubes we found a significant reduction in total protein content. Using functional assays we observed protein degradation elevation in the first 24 h while synthesis was maintained during this period and then significantly decrease at 48 h. These results demonstrate a temporal regulation of protein homeostasis, whereby elevated protein degradation is followed by a reduction in synthesis. These results are comparable to the cellular adaptation seen during development of muscle atrophy.

1. Introduction

Muscle atrophy is a common clinical manifestation occurring in a variety of hypoxemic conditions, including chronic obstructive pulmonary disease (COPD) [1–3]. In these subjects, peripheral muscle wasting decreases functional capacity and increases mortality [4]. We and others have hypothesized that hypoxemia and the resulting tissue hypoxia is among the factors that might contribute for the development of muscle atrophy [3,5]. However, this statement has not been directly addressed.

Muscle mass maintenance relies on a fine regulation between contractile protein degradation and synthesis. In wasting conditions, contractile protein degradation is mostly achieved through the activation of the ubiquitin-proteasome (Ub-P'some) [6]. Since this system is unable to directly break up the actomyosin structure found in myofibrils [7], a preliminary rate-limiting step is required to fragment actomyosin into substrates that will be degraded by the Ub-P'some. Among these substrates, actin fragments represent a reliable marker for contractile protein degradation [8].

The major cascade involved in protein synthesis is the insulin-like growth factor-1 (IGF-1)/phosphatidylinositol-3 kinase (PI3K)/Akt pathway. Akt is a kinase which, upon phosphorylation, ensures cell survival, differentiation and protein synthesis by modulating the activity of numerous targets including glycogen synthase kinase-3 (GSK-3) [9].

In this study, we aimed to provide direct evidences demonstrating that hypoxia is involved in the instigation of cellular events compatible with the initiation and the development of muscle atrophy. We hypothesized that hypoxic exposure of L6 myotubes would result in an elevation of contractile protein degradation and the activation of the Ub-P'some system with a concomitant reduction in protein synthesis and activity of the IGF-1/PI3K/Akt pathway.

2. Materials and methods

2.1. Cell culture and hypoxic exposures

L6 rat myoblasts (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) with 1% glucose (HyClone, Logan, UT, USA), 10% fetal bovine serum, penicillin 50 U/ml, and streptomycin 50 μg/ml (HyClone). Confluent myoblasts (80%) were placed in differentiation medium made of DMEM with 1% glucose, 2% horse serum, penicillin 50 U/ml, and streptomycin 50 μg/ml. After 4 days, differentiated myotubes were transferred to a chamber maintained at 1% O_2, 5% CO_2, 94% N_2 (ProOx system, BioSpherix, Redfield, NY, USA). Prior to hypoxic exposure, differentiation medium was replaced with medium preconditioned overnight in 1% O_2 atmosphere. After designated exposures, hypoxic cells were lysed in 1% O_2 atmosphere inside a C-Shuttle Glovebox (Biospherix) according to the subsequent protocols.

Abbreviations: COPD, chronic obstructive pulmonary disease; Ub-P'some, ubiquitin-proteasome; DMEM, Dulbecco's modified Eagle's medium; E3, ubiquitin ligase; EDTA, ethylenediamine tetraacetic acid; GSK-3, glycogen synthase kinase-3; IGF-1, insulin-like growth factor-1; PBS, phosphate buffered saline; PI3K, phosphatidylinositol-3 kinase

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2.2. Cell viability assessments

Cell stress induced by hypoxia was assessed by measuring lactate dehydrogenase (LDH) leakage [10] using LDH-Cytotoxicity Assay Kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. For each experimental condition, percentage of cytotoxicity was calculated using the following formula: (LDH media/(LDH media + LDH cells)) × 100. General appearance of myotubes was examined using a Nikon Eclipse TE300 inverted microscope (Nikon Corporation, Tokyo, Japan) and images were captured with a Nikon 950 digital camera (Nikon Corporation). Number of nucleus was assessed using 4′,6-diamidino-2-phenylindole (DAPI) staining (Invitrogen, Carlsbad, CA, USA). Briefly, myotubes were grown on four wells Lab-Tek™ chamber slides (Thermo Fisher Scientific, Waltham, MA, USA). After designated expositions, cells were fixed with 2% paraformaldehyde for 15 min and DAPI was added for 2 min at a final concentration of 30 nM in phosphate buffered saline (PBS) 1X supplemented with 1% bovine serum albumin. Images were captured using a Nikon Eclipse E600 microscope (Nikon Corporation) and all nuclei from six random separate fields were counted.

2.3. Actomyosin breakdown measurements

The 14-kDa actin fragment accumulation, an indicator of actomyosin degradation, was measured as previously described [8]. Cells were scrapped in a hypotonic lysis buffer [5 mM Tris–HCl pH 8.0, 1 mM β-mercaptoethanol, 1% glycerol, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM EGTA, protease inhibitor cocktail set III (EMD biosciences, San Diego, CA, USA)]. After centrifugation (13 000 rpm, 4 °C, 10 min), supernatant was collected for detection of accumulated 14-kDa actin fragment by Western blot using an anti-actin antibody which recognizes the carboxy-terminal 11 amino acids (1:500, #A2066; Sigma–Aldrich, St. Louis, MO, USA). Anti-actin antibody was then detected using a secondary antibody coupled with horseradish peroxidase (1:5000, #7074; Cell Signaling Technology Inc.). Results were normalized to tubulin (1:20 000; Sigma–Aldrich).

2.4. Proteasome activity assays

Proteasome activity assays were performed using a modified protocol [11]. Briefly, cells were washed in ice-cold PBS and scrapped in lysis buffer [50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT, 200 μM PMSF, protease inhibitor cocktail set III (EMD biosciences)]. Lysate was grinded on ice and centrifuged (10 000×*g*, 4 °C, 20 min). Supernatant was collected and centrifuged (100 000×*g*, 4 °C, 45 min). Resulting pellet was resuspended [50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 20% glycerol] and 10 μg of protein was added to 50 μl of reaction buffer [50 mM Tris–HCl pH 8, 10 mM $MgCl_2$, 2U Apyrase (Sigma–Aldrich)] containing either 100 μM Suc-LLVY-AMC or 600 μM Z-LLE-AMC (Sigma–Aldrich) to quantify chymotrypsin-like and caspase-like proteasomal activities, respectively. After 15 min of incubation at 37 °C, fluorescence was measured (excitation 360 nm, emission 460 nm) (Fluoroskan Ascent; Labsystems, Helsinki, Finland).

2.5. Real-time PCR

Total RNA was isolated using TRIzol® Reagent (Invitrogen) and its quantity and purity were determined by spectrophotometry. Reverse transcription was performed using Quantitect™ Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Real-time PCR was performed with the Quantitect™ SYBR® Green PCR Kit (Qiagen) in an Opticon® 2 (MJ Research, Waltham, MA, USA). 18S mRNA was used as an internal control. Primers used were (sense and antisense, 5′–3′): Atrogin-1 gtccagagagtcggcaagtc, gtcggtgatcgtgagacctt (141pb); MuRF1 tgaccaaggaaaacagccaccag, tcactcttcttctcgtccaggatgg (88pb) [12]; 18S acggaagggcaccaccagga, caccaccacccacggaatcg (127pb) [13]. Real-time PCR were analyzed using the $2^{-\Delta\,\Delta}C_T$ method [14].

2.6. Protein synthesis measurements

Total protein synthesis was appraised by measuring cellular incorporation of L-[2,3,4,5,6-^{3}H]Phenylalanine (Amersham, Buckinghamshire, UK). Myotubes grown in a six wells plate were incubated in differentiation medium containing L-[2,3,4,5,6-^{3}H]-Phenylalanine (0.5 μCi/ml). Controls with the proteasome inhibitor epoxomicin (10 nM) were used to inhibit proteolysis. In a subsequent step, cells were washed in ice-cold PBS and incubated overnight in a lysis buffer [PBS + 1% SDS] at 4 °C. L-[2,3,4,5,6-^{3}H]-Phenylalanine uptake was measured in the lysates using a Tri-Carb® 2100TR liquid scintillation counter (Perkin–Elmer Life Sciences, Boston, MA, USA).

2.7. Western blotting

Myotubes were washed with ice-cold PBS and incubated on ice in lysis buffer [10 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 2% Triton X-100, protease inhibitor cocktail set III (EMD biosciences), phosphatase inhibitor cocktail 1 and 2 (Sigma–Aldrich)] for 30 min. Western blots were performed with 60 μg of whole cell extracts using standard SDS–PAGE procedures. After transfer on a nitrocellulose membrane, immunoblotting with anti-Akt (1:2000), anti-phospho-Akt (Ser473) (1:1000), anti-GSK-3β (1:1000) or anti-phospho-GSK-3β (1:1000) (Cell Signaling Technology, Danvers, MA, USA) was performed. Proteins of interest were detected using a secondary antibody coupled with horseradish peroxidase (1:5000, #7074; Cell Signaling Technology Inc.). Results were normalized to tubulin (1:20 000; Sigma–Aldrich).

2.8. Akt kinase activity assays

Akt kinase activity was evaluated using an Akt Activity Immunoassay Kit (EMD biosciences) according to the manufacturer's instructions.

2.9. Statistical analyses

All statistical analyses were performed using JMP® 7.0 (SAS Institute, Cary, NC, USA). Comparisons of experimental conditions were carried out by ANOVA. *P*-value <0.05 was considered significant.

3. Results

3.1. Cell viability is minimally affected in hypoxic myotubes

We first evaluated the hypoxic stress on myotubes by measuring LDH leakage in the culture media as an indicator of membrane integrity. In hypoxic myotubes, we found a significant increase in LDH leakage (31.5 ± 3.4%, $n = 3$; $P < 0.05$) after 48 h of exposure when compared to matching normoxic controls (Fig. 1A). Insignificant change was observed at 24 h (2.5 ± 1.6%, $n = 3$; $P > 0.05$). Despite an increase in LDH leakage, the main cellular architecture of myotubes is preserved even if minor to moderate structural changes are observed after 48 h of hypoxic exposure (Fig. 1B). Finally, nuclei number present at 48 h in hypoxic myotubes (Fig. 1C) was decreased by 11.3% ($n = 6$, $P < 0.05$). Overall, these results indicate that our hypoxic protocol has a sig-

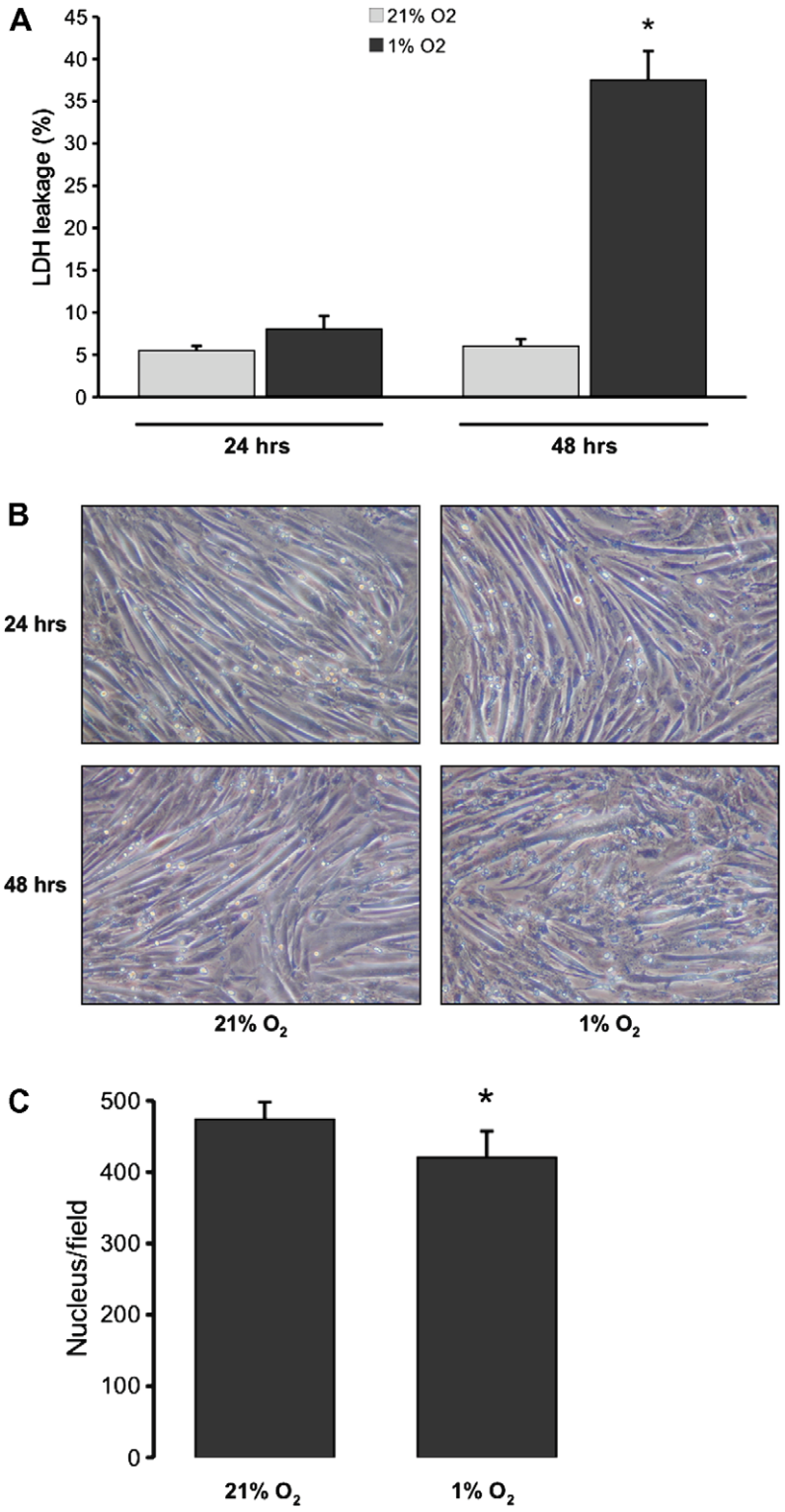

Fig. 1. Chronic hypoxic exposure induces cellular injuries and slightly decreased cell viability. (A) Myotubes were either exposed to 21% or 1% O_2 for 24 h or 48 h. LDH leakage was measured as an indication of membrane integrity. Results are mean ± S.E.M. of three independent experiments. $^*P < 0.05$. (B) Following exposure to 21% or 1% O_2 for 24 h or 48 h, cells were visualized on an inverted microscope as described in Section 2 and typical observations are depicted (200×). (C) Myotubes were stained with DAPI following 48 h incubation to 21% or 1% O_2. Total nuclei per field (200×) were counted and results are mean ± S.E.M. of six random fields per condition. $^*P < 0.05$

nificant impact on cell membrane integrity after 48 h of hypoxic exposure (LDH leakage) but minor effect on myotubes viability (nuclei number). Based on this observation, we decided to further investigate the direct effects of chronic hypoxia on protein homeostasis in our model.

3.2. Total protein is negatively affected by hypoxia

We evaluated the impact of hypoxia on myotubes protein content. To do so, we directly measured the total protein concentration in our protein extracts using a protein assay based on the

Lowry protocol (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). After 24 h, we observed a 24% reduction of total protein concentration in cells exposed to 1% O_2 compared to 21% O_2 (3.29 μg/μl ± 0.21 and 4.33 μg/μl ± 0.35, respectively; $n = 11$, $P < 0.05$). This finding reinforced the notion that chronic hypoxia could affect both protein degradation and synthesis.

3.3. Protein degradation increases in hypoxic myotubes

We assessed protein degradation in myotubes using two distinct methods. First, we quantified the accumulation of a 14-kDa actin fragment. After 12 h of hypoxic exposure, myotubes had a 134% increase in actin fragment compared to cells exposed at 21% O_2 (Fig. 2). This increase was maintained after 24 h (Fig. 2). Since the Ub-P'some system plays a central role in muscle protein degradation [6], we next evaluated chymotrypsin-like and caspase-like proteasomal activities in our model. In myotubes exposed to 1% O_2 for 24 h, both activities were respectively increased by 38% ($P < 0.05$) and 86% ($P < 0.05$) compared to cells exposed to 21% O_2 (Fig. 3).

Atrogin-1 and MuRF1 are two muscle specific ubiquitin ligases (E3s) upregulated in various muscle atrophy models [15,16]. Their role is to attach activated ubiquitin to targeted substrates that will be degraded by the proteasome. We evaluated their mRNA expression at various time points in myotubes exposed to 1% O_2. Atrogin-1 level was significantly increased by 100% ($P < 0.05$) after 24 h in hypoxic conditions whereas MuRF1 level did not change (Fig. 4).

Taken as whole, these results are indicative of an increased potential for protein degradation in hypoxic myotubes. To further explore the effect of hypoxia on intracellular protein balance, we tested the hypothesis that protein synthesis was altered in hypoxic conditions.

3.4. Protein synthesis is decreased in hypoxic myotubes

To directly measure total muscle protein synthesis, we assessed L-[2,3,4,5,6-3H]Phenylalanine uptake at 21% and 1% O_2. After 24 h, a non-significant decrease of 5% in L-[2,3,4,5,6-3H]Phenylalanine uptake was observed in hypoxic conditions (data not shown). However, after 48 h exposure, L-[2,3,4,5,6-3H]Phenylalanine incorporation was significantly decreased by 49% ($P < 0.05$) in hypoxic myotubes (Fig. 5). Only protein synthesis was assessed since similar rate of incorporation was measured when epoxomicin was added to culture medium (Fig. 5). As a result, these findings are indicative of impaired protein synthesis in hypoxic conditions.

Because the IGF-1/PI3K/Akt pathway plays a major role in protein synthesis, we sought to evaluate its activity in our model. There was a non-significant trend towards a decrease in the phospho-Akt/Total Akt ratio after 24 h at 1% O_2 (Fig. 6A). When phosphorylated and total Akt were analyzed separately, we found that both forms were diminished at 1% O_2 (57% and 40%, respectively), although only the decrease in the phosphorylated form reached statistical significance (Fig. 6A). To confirm these results, Akt kinase activity was directly assessed and shown to be significantly reduced after 24 h of exposure to 1% O_2 as revealed by a lower phosphorylation level of recombinant GSK-3α (Fig. 6B). We verified the phosphorylation level of GSK-3β, a downstream target of Akt, after 24 h of hypoxic exposure. Phospho-GSK-3β/Total GSK-3β ratio was decreased by 36% ($P < 0.05$) in myotubes exposed to 1% O_2 (Fig. 7).

Taken as whole, these results are indicative of a reduced potential for protein synthesis in hypoxic myotubes.

4. Discussion

Cells exposed to hypoxia turn on specific processes that allow proper adaptation to survive [17]. This study provides, for the first time, direct evidences linking hypoxia to cellular responses compatible with the initiation and the development of muscle atrophy.

A change in either protein synthesis or degradation can have dramatic effects on muscle mass. The significant decrease in total protein found in hypoxic myotubes was the first indication that protein homeostasis was perturbed. This study demonstrates that hypoxia act directly on muscle cells by sequentially enhancing protein degradation and reducing protein synthesis.

4.1. Proteolysis activation

We found an increase in actomyosin breakdown, as revealed by the significant accumulation of the 14-kDa actin fragment. This

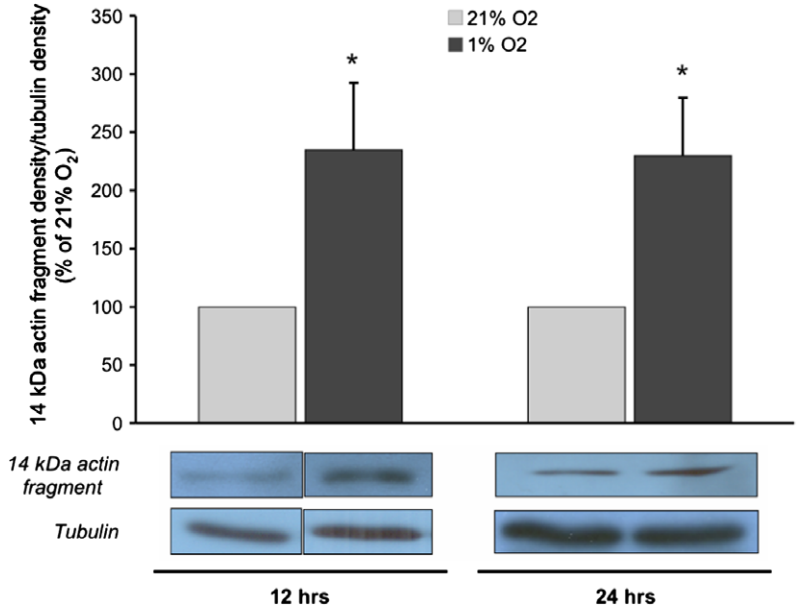

Fig. 2. Actomyosin fragmentation is accentuated in hypoxia. Myotubes were either exposed to 21% or 1% O_2 for 12 h or 24 h. The accumulation of the 14-kDa actin fragment was assessed by Western blotting. The 21% O_2 condition was set at 100% and compared to 1% O_2 condition. Results are reported as a ratio of the actin fragment density on tubulin density and are mean ± S.E.M. of six independent experiments. * $P < 0.05$

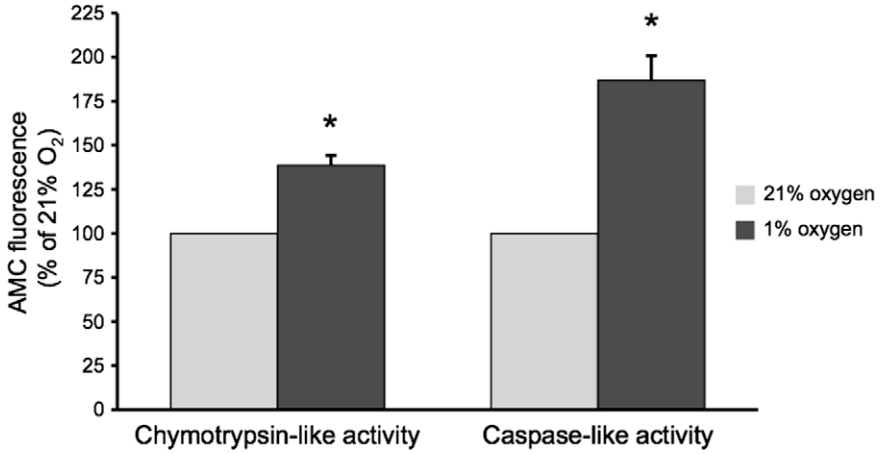

Fig. 3. Proteasomal activities are increased in hypoxia. Proteasomal units were isolated from myotubes exposed 24 h at 21% O_2 (light gray) or 1% O_2 (dark gray) and the chymotrypsin-like and caspase-like activities were determined as described in Section 2. The 21% O_2 condition was set at 100% and compared to 1% O_2 condition. Results are reported as mean ± S.E.M. of three independent experiments. * $P < 0.05$ versus 21% O_2 condition.

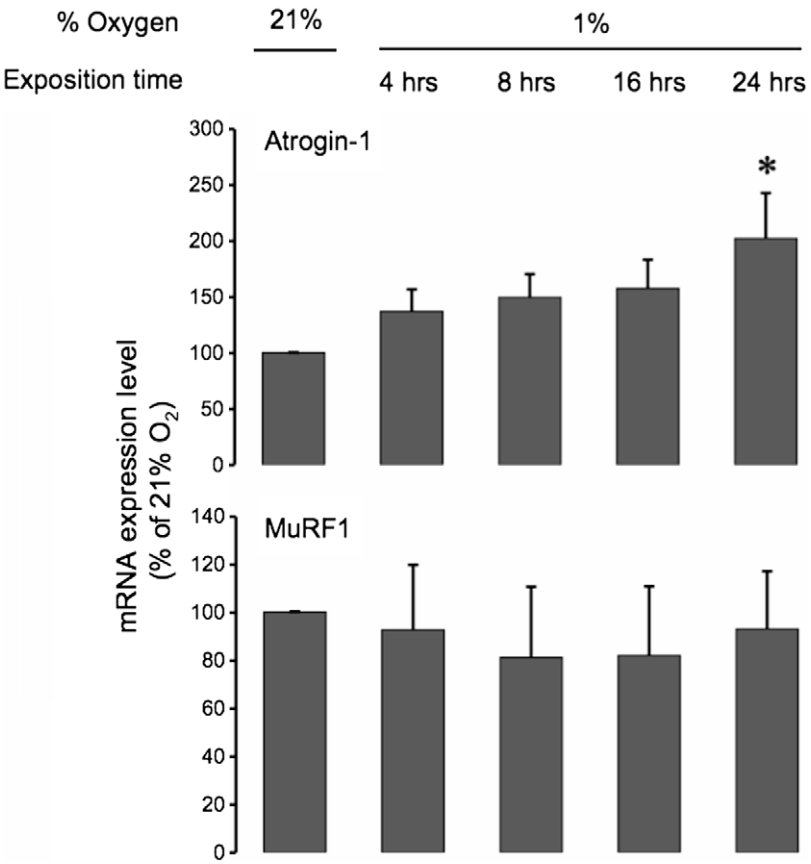

Fig. 4. Hypoxia increases Atrogin-1 mRNA expression. Myotubes were either exposed to 21% O_2 or 1% O_2 for the indicated period of time. Atrogin-1 and MuRF1 mRNA expression levels were measured using 18S as internal control. The 21% O_2 condition was set at 100% and compared to hypoxic exposures. Results are reported as mean ± S.E.M. of four independent experiments. * $P < 0.05$ versus 21% O_2 condition.

observation is central since actomyosin is the main protein complex found in myofibrils, the fundamental contractile unit of muscle cell. In a subsequent step, we directly assessed the activity of the Ub-P'some system in hypoxic conditions. We found an increase in proteasomal activity, as chymotrypsin-like and caspase-like activities were both significantly up-regulated. Higher proteasomal activity is preceded by ubiquitination of targeted substrates and Atrogin-1 and MuRF1 are key regulators of this process. Under hypoxic conditions, up-regulation of Atrogin-1 mRNA expression level but not of MuRF1 was observed. Interestingly, the Atrogin-1 mRNA level reached in hypoxic conditions is comparable to the level previously reported in humans with muscle atrophy [18]. The reported discordant regulation of Atrogin-1 and MuRF1 is not unique and has been observed in both cellular [19] and human [20] models. This observation suggests that Atrogin-1 could be the preferential E3 activated in hypoxic muscle cells.

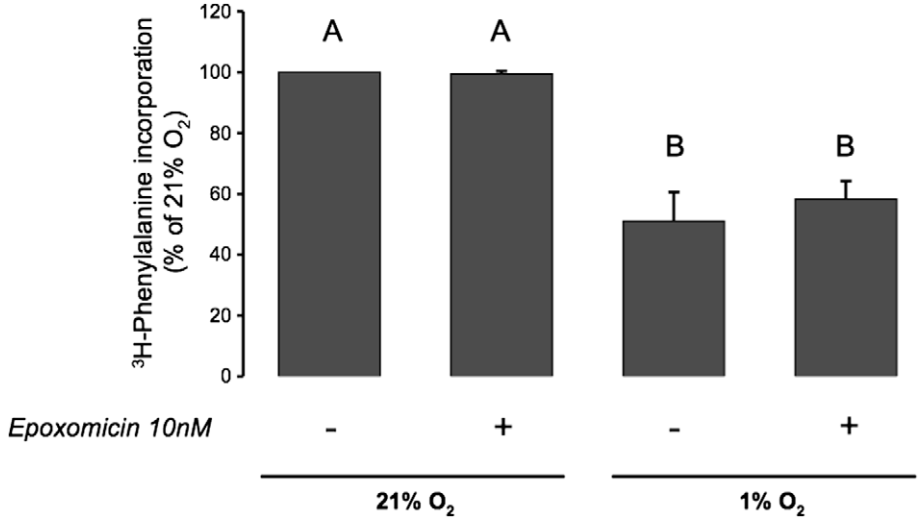

Fig. 5. Total protein synthesis is decreased in hypoxia. Total protein synthesis was evaluated by measuring the L-[2,3,4,5,6-^{3}H]Phenylalanine uptake in myotubes exposed either to 21% or 1% O_2 for 48 h. The 21% O_2 condition without epoxomicin was set at 100% and data are stated as relative changes compared to this condition. Results are reported as mean ± S.E.M. of at least three independent experiments. $P < 0.05$ when the letters over the columns are different.

4.2. Diminution of the protein synthesis activity

Another key result of the present study is the 49% reduction in total protein synthesis in hypoxic myotubes. Although we observe an increase in LDH leakage at 48 h, this method is an imperfect indicator of cell mortality in multinucleated cells. The 11.3% decrease in total nuclei suggests that leaking myotubes are still viable. The presence of a substantial amount of transcriptional active nuclei in hypoxic myotubes likely sustains cell functions. Moreover, the altered cell viability reported at 48 h cannot entirely explain the 49% decline observed in protein synthesis including the early alteration seen in IGF/PI3K/Akt pathway. Although we are confident with the interpretation of these results, 48 h of hypoxic exposure on L6 myotubes is likely the limit of this model to study protein homeostasis. Thus, in addition to the increased protein degradation, there is a subsequent reduction in the regeneration of muscle protein content under hypoxic conditions. This adaptative response to hypoxia could explain the loss of 26% of total protein content observed in hypoxic myotubes and support the notion that hypoxia has the potential to contribute to muscle atrophy. To further describe the effects of hypoxia on muscle cells, we measured the activation state of the IGF/PI3K/Akt pathway. Upon phos-

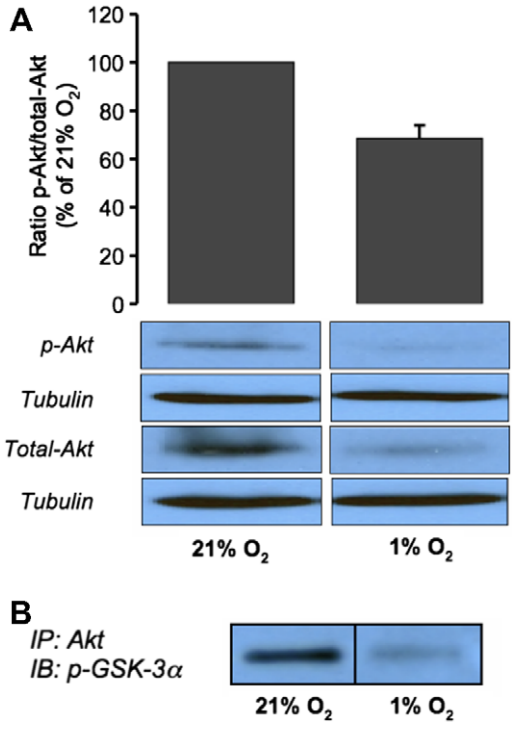

Fig. 6. Akt kinase activity is impaired in hypoxia. (A) Whole cell extracts were blotted for phosphorylated and total Akt after 24 h exposition to 21% or 1% O_2. The 21% O_2 condition was set at 100% and compared to hypoxic exposures. Data are presented as phospho-Akt/total Akt ratios. Results are reported as mean ± S.E.M. of three independent experiments. (B) Following 24 h exposition to 21% or 1% O_2, Akt was immunoprecipitated and its kinase activity was evaluated by measuring its ability to phosphorylate recombinant GSK-3α. A Western blot against phospho-GSK-3α was performed. This experiment was repeated three times and a representative blot is shown.

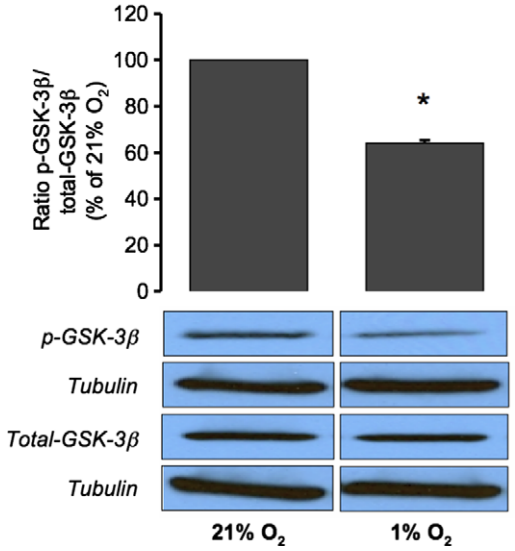

Fig. 7. GSK-3β phosphorylation level is diminished in hypoxia. Myotubes were incubated to 21% or 1% O_2 for 24 h. Whole cell extracts were blotted for phospho-GSK-3β and total GSK-3β. The 21% O_2 condition set at 100% and compared to hypoxic exposures. Data are presented as phospho-GSK-3β/total GSK-3β ratios. Results are reported as mean ± S.E.M. of three independent experiments. * $P < 0.05$.

phorylation, Akt is activated and acts as an important modulator of the generation of signals in favor of protein synthesis [9]. In hypoxic conditions, we found a decrease in Akt kinase activity, suggesting that the IGF/PI3K/Akt pathway activity is reduced in hypoxia. The total Akt reduction is probably contributing to this low synthesis state in hypoxic myotubes. Interestingly, this result has also been reported in peripheral muscle of subjects with muscle atrophy [18]. Potential explanations for this reduction could be the inability to properly translate Akt mRNA into functional protein or post-translational modifications of Akt affecting its stability and promoting its degradation [21]. GSK-3β, a target of Akt, was also hypophosphorylated after 24 h of hypoxic exposure. This observation also supports the concept that IGF-1/PI3K/Akt pathway activity is lowered in hypoxic myotubes.

4.3. Contractile protein homeostasis: integration

Cells undergoing hypoxic stress modify their form and function to maintain homeostasis. Of interest is the size reduction that occurs in organisms adapting to low O_2 environment [17]. Consistent with this notion, protein content was decreased in hypoxic myotubes, suggesting that these cells are undergoing atrophy. Based on our results, it is interesting to note that both protein degradation and synthesis are contributive to this cell remodeling, in an asynchronous manner. We propose a model conceptualizing this notion into two successive intricate moments (Fig. 8). When cells are exposed to hypoxia, increased protein degradation is the first cell response. This situation, where protein degradation outweighs protein synthesis, leads to a net protein loss (T1). Further progression in time conducts to a decrease in protein synthesis below the level observed in normoxia, leading to a further reduction in protein content in hypoxic myotubes and an inability to synthesize sufficient proteins to overcome the previous loss (T2).

Overall, these results are in strong agreement with the notion that hypoxia is acting as a catabolic trigger on muscle cells. Muscle volume balance is an important biological issue in living organisms and better understanding of the mechanisms leading to muscle atrophy is necessary to reverse its adverse effects. Here we demonstrate that hypoxia is an additional factor to consider in this complex equation.

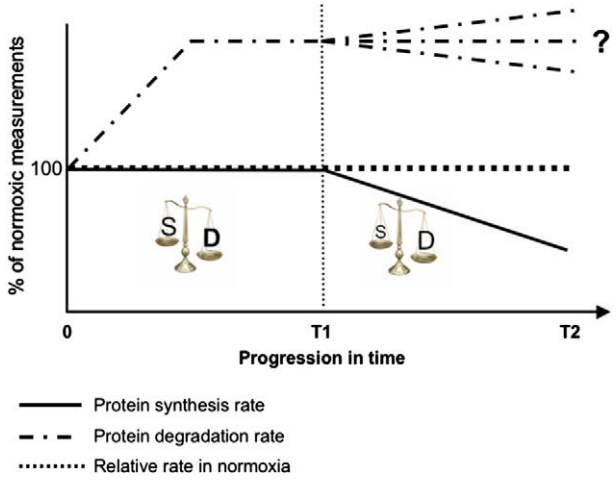

Fig. 8. Protein degradation and synthesis are sequentially activated in hypoxic myotubes. Protein degradation is increased in the first part (T1) of chronic hypoxic exposure. This situation brings an imbalance in protein homeostasis where protein degradation outweighs protein synthesis, which leads to a net protein loss. When the hypoxic exposure continues (T2), protein synthesis is decreased below normoxic level in hypoxia, still creating an imbalance in protein homeostasis.

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